

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Assistant Commissioner for Patents
Washington, D.C. 20231

Atty. Dkt.: 159-62

Date: November 1, 2000

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Applicant: TAMURA et al.
Entitled: **METHOD FOR DETERMINING CRP USING PHOSPHORYLCHOLINE**

and including attachments as noted below:

☒ Declaration (no signature of 6th Inventor, Kichitaro KAWAGUCHI, who was deceased on October 12, 1999),

☒ Abstract

30 pages of translation of specification and claims (including 9 numbered claims) into English, and
8 sheets of accompanying drawings.

☒ Record & return the attached assignment to the undersigned.

☒ Priority is hereby claimed under 35 U.S.C 119 based on the following foreign applications, the entire content of which is hereby incorporated by reference in this application:

Application Number	Country	Day/Month/Year Filed
55352/1999	JAPAN	3 March 1999
PCT/JP00/01268	JAPAN	3 March 2000

, respectively.

☐ Certified copy(ies) of foreign application(s) is/are attached.

☒ Please amend the specification by inserting before the first line --This is a continuation of PCT application PCT/JP00/01268, filed 3 March 2000, the entire content of which is hereby incorporated by reference in this application.--

☒ Priority is hereby claimed under 35 U.S.C 120/365 based on the following prior PCT applications designating the U.S., the entire content of which is hereby incorporated by reference in this application:

Application Number	Country	Day/Month/Year Filed
PCT/JP00/01268		3 March 2000

☐ This application is based on the following prior provisional application(s):

Application No.	Filing Date
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☐ , respectively, the entire content of which is hereby incorporated by reference in this application, and priority is hereby claimed therefrom.

☐ Please amend the specification by inserting before the first line: --This application claims the benefit of U.S.

☐ Provisional Application No. , filed , the entire content of which is hereby incorporated by reference in this application.--

☐ Verified Statement attached establishing "small entity" status (Rules 9 & 27)

☐ The Examiner's attention is directed to the prior art cited in the parent application by applicant and/or Examiner for the reasons stated therein.

☒ Preliminary amendment to claims (attached hereto), to be entered before calculation of the fee below.

☒ Also attached: **PTO-1449/ International Search Report/ Declaration of**

Translation with Family Register**FILING FEE IS BASED ON CLAIMS AS FILED LESS ANY HEREWITH CANCELED**

Basic Filing Fee		\$	710.00
Total effective claims	9 - 20 (at least 20) = 0 x \$ 18.00	\$	0.00
Independent claims	3 - 3 (at least 3) = 0 x \$ 80.00	\$	0.00
If any proper multiple dependent claims now added for first time, add \$270.00 (ignore improper)		\$	0.00
	SUBTOTAL	\$	710.00
If "small entity," then enter half (1/2) of subtotal and subtract		-\$	(0.00)
	SECOND SUBTOTAL	\$	710.00
Assignment Recording Fee (\$40.00)		\$	40.00
	TOTAL FEE ENCLOSED	\$	750.00

Any future submission requiring an extension of time is hereby stated to include a petition for such time extension.

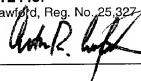
The Commissioner is hereby authorized to charge any deficiency in the fee(s) filed, or asserted to be filed, or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our **Account No. 14-1140**. A duplicate copy of this sheet is attached.

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Signature: _____



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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

TAMURA et al.

Atty. Ref.: **159-62**

Serial No. **(To Be Assigned)**

Group:

National Phase of **PCT/JP00/01268**

Filed: **November 1, 2000**

Examiner:

For: **METHOD FOR DETERMINING CRP USING
PHOSPHORYLCHOLINE**

* * * * *

November 1, 2000

Assistant Commissioner for Patents
Washington, DC 20231
Sir:

PRELIMINARY AMENDMENT

Prior to calculation of the filing fee and in order to place the above identified application in better condition for examination, please amend the claims as follows:

IN THE CLAIMS

Claim 4, line 1, change "any one of Claims 1 to 3" to --Claim 1--.

Claim 7, line 1, delete "or 6".

REMARKS

The above amendments are made to place the claims in a more traditional format.

Respectfully submitted,

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U.S. PATENT APPLICATION

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Invention: METHOD FOR DETERMINING CRP USING PHOSPHORYLCHOLINE

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SPECIFICATION

METHOD FOR DETERMINING CRP USING PHOSPHORYLCHOLINE

5 TECHNICAL FIELD

TECHNICAL FIELD

BACKGROUND OF THE INVENTION

- 1 -

bacterial infection, inflammation and for post-surgery prognosis.

Although the biological role of CRP has not yet been completely elucidated, it has gradually been ascertained that CRP exerts various pro-inflammatory host-protection activities including complement activation [Richards, R. L., et al., (1997) Proc. Natl. Acad. Sci. USA 74, 5672-5676; Richards, R. L., et al., (1979) J. Immunol. 122, 1185-1189; Miyazawa, K., and Inoue, K. (1990) J. Immunol. 145, 650-654; Jiang, H., et al., (1991) J. Immunol. 146, 2324-2330; Wolbink, G. J., et al., (1996) J. Immunol. 157, 473-479] and lymphocyte opsonization [Mortensen, R. F., et al., (1976) J. Immunol. 117, 774; Edwards, W. M., et al., (1982) J. Immunol. 128, 2498; Szalai, A. J., et al., (1995) J. Immunol. 155, 2557-2563]. It is likely that these activities make contributions to non-specific host protection in the pre-immune phase of an inflammatory reaction.

In order to diagnose bacterial infection, inflammation and to assist in post-surgery prognosis, it is necessary for an acute phase protein level to be assayed accurately not only in an acute phase but also for it to be assayed accurately when a person is in a healthy state. CRP is an acute phase protein most sensitive and most highly reactive to extraneous stimuli [Morley, J. J., and Kushner, L. (1982) Ann. N. Y. Acad. Sci. 389, 406-418]. Therefore, the ability to accurately assay CRP levels in a healthy individual, will enable the early

stages of infection to be diagnosed more accurately. The early increase of CRP level at acute phase essentially needs to be detected in a rapid and accurate manner in order to monitor therapeutic treatment and to diagnose prognosis, wherein the detection requires a highly sensitive method capable of assaying serum CRP level within the normal range.

It is known that using a nephelometric immunoassay method [Yamamoto, S., et al., (1994) Vet. Quarterly 16, 74-77; Yamamoto, S., et al., (1993) Vet. Immunol. Immunopathol. 36, 257-264] or latex agglutination test [Sarikaputi, M., et al., (1992) Jap. J. Vet. Res. 40, 1-12; Yamada, T., et al., (1993) Ann. Clin. Biochem. 30, 72-76] CRP levels can be determined within a range of 1 to 1000 µg CRP/ml and within a range of 10 to 400 µg CRP/ml, respectively, in a simple manner. These ranges include the typical serum range (10 to 100 µg CRP/ml) in an acute phase. Since blood CRP concentration in a healthy individual is below about 350 ng/ml as stated above, however, the aforementioned methods are incapable of assaying CRP concentrations at within a normal range. Although these methods have been used widely at clinical tests, they are not sufficiently sensitive to detect early CRP level elevation at the beginning of an acute phase. Thus, assay results are simply expressed as "negative", "positive" or "elevation". In addition to the assay of CRP level at peak in acute phase, precise assay of normal CRP level or CRP level at the onset of acute phase is

extremely useful so as to enhance the precision of diagnosis.

Compared with the aforementioned methods, fluorescent immunoassay (FIA) [Siboo, R., and Kulisek, E. A. (1976) J. Immunol. Methods 23, 59-67] and radioimmunoassay [Claus, D. R., et al., (1976) J. Lab. Clin. Med. 87, 120-128] are considerably more sensitive. However, they are not sufficiently sensitive to effect assay of serum CRP in a healthy individual. Enzyme-linked immunosorbent assay (ELISA) [Lamb, W. R., et al., (1991) Med. Lab. Sci. 48, 201-205; Shields, M., et al., (1991) J. Immunol. Methods 141, 253-261; Yamamoto, S., et al., (1994) Vet. Quarterly 16, 74/77] is based on the same principle as those employed in both FIA and RIA, and represents a development of these methods. ELISA is a quantitative assay method, generally having greater sensitivity than that of RIA. When CRP concentration is assayed, however, no significant difference is found between the CRP range assayed by ELISA and the range assayed by RIA. Specifically, compared with the range of 8 to 800 ng CRP/ml as detected by RIA [Claus, D. R., et al., (1976) J. Lab. Clin. Med. 87, 120-128], the range detected by ELISA is 10 to 100 ng CRP/ml [Unten, S. K., and Hokama, Y. (1987) J. Clin. Lab. Anal. 1, 136-139]. These results are indicative of the fact that ELISA for CRP assay needs to be greatly modified.

SUMMARY OF THE INVENTION

The purpose of the present invention is to provide a method for rapidly determining CRP in a sample which

includes not only a CRP concentration in the serum occurring in an acute phase, but also serum CRP which is within the normal range, with a high degree of sensitivity.

- 5 Another purpose of the present invention is to develop a method for rapidly assaying CRP in a sample with a high degree of sensitivity and which receives no influence from frompentraxin family proteins having high homology to CRP present in serum, using phosphorylcholine (PC), and wherein the method is applicable in clinical diagnosis.

BRIEF DESCRIPTION OF THE DRAWINGS

- Fig. 1 shows a method for determining CRP using Eu^{3+} -labeled probe, wherein (A), (B) and (C) show the results of the assay of CRP concentration by an antibody-antibody method, antibody-PC method and PC-antibody method, respectively;

- Fig. 2 shows standard curves for CRP assay, as analyzed by using the antibody-antibody method (A), the antibody-PC method (B) and the PC-antibody method (C);

Fig. 3 shows the results of CRP assay in 100-fold diluted human serum obtained by using each of the above methods;

- Fig. 4 shows the results of CRP assay in 100-fold diluted serum by the antibody-antibody method (A) and the antibody-PC method (B);

Fig. 5 shows the quantitative precipitation of CRP with PC_{38} -BSA;

Fig. 6 shows the comparison in CRP-binding activity between PC₅₆- and PC_γ-BSA;

Fig. 7 shows the inhibition of the PC-binding activity of CRP by water-soluble PC; and

5 Fig. 8 shows specific and non-specific CRP bindings according to the antibody-PC method (B).

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have studied extensively various CRP assay methods. As a result of such studies,
10 the inventors have found that proteins of the pentraxin family, such as amyloid A and amyloid P, are present in human serum and these proteins react with anti-CRP antibodies. It is suggested that one of the factors inhibiting the elevation of the assay sensitivity for the
15 assay of CRP concentration by ELISA is the reactivity of the proteins with anti-CRP antibodies and that it is therefore very difficult to accurately determine CRP in, for example, human serum samples.

Focussing on the reactivity of CRP with C-
20 polysaccharide existing in the capsule of Diplococcus pneumonia to bind to the phosphorylcholine group of the C-polysaccharide, the present inventors have determined the possibility of realizing highly sensitive assay of CRP concentration, by utilizing the specific binding of CRP to
25 phosphorylcholine.

In accordance with the present invention, an assay is thus performed by a sandwich assay method using the specific binding of CRP to phosphorylcholine (PC). In a

preferred embodiment, PC is labeled and then used. In such a case, PC may be directly labeled or indirectly labeled by conjugating another molecule to PC, wherein said molecule mediates a labeling means. Additionally, PC
5 may be labeled with a radioactive labeling means or with a non-radioactive labeling means. Further, these labeling means are not limited to currently available labeling means.

As a radioactive labeling means for labeling PC,
10 conventional radioisotopes such as ^{125}I and ^{32}P can be used. As a non-radioactive labeling means for labeling PC, any of the following methods can be used: methods using enzymes, such as alkali phosphatase (AP), horse radish peroxidase, β -galactosidase and glucose oxidase; methods
15 using luminescence from luciferin with luciferase; and methods using fluorescent dyes such as fluorescein and lanthanide. However, it should be noted that since the use of radioactive materials may not be preferable in conducting examination on site in clinical practice, the
20 use of a non-radioactive labeling means is generally preferable. In one embodiment of the invention, lanthanum-series elements (lanthanides) are particularly used as fluorescent substances.

Lanthanides have been drawing attention in recent
25 years as fluorescent probes for use in assaying biologically active compounds (for example, enzyme, lectin, receptor, and the like) and for histochemical research studies. Lanthanides emitting fluorescence, samarium

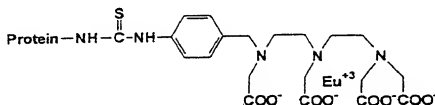
(Sm³⁺), europium (Eu³⁺), and terbium (Tb³⁺) are known [Saito, K., et al., (1998) Anal. Biochem., 258, 311-314; Elliot, C. T., et al., (1994) Analyst, 119, 2565-2569; Pettersson, K., et al., (1993) Clin. Chem., 39, 2084-2089; Rothlin, M. A., et al., (1993) Cancer, 71, 701-707; Samiotaki, M., et al., (1997) Anal. Biochem., 253, 156-161]. One of the advantages of utilizing fluorescence emitted from such lanthanides is that sensitivity at the same level as or at a level higher than that recovered by the methods utilizing radioisotopes can be obtained.

Any lanthanide which emits fluorescence can be utilized as fluorescent substance in labeling PC with lanthanides in the present invention. Among them, the use of Eu³⁺ is preferable. Eu³⁺ exhibits high sensitivity, has a long fluorescence life (far longer than the mean background fluorescence by FIA), and, in addition, the shelf life of Eu³⁺ labeling reagents is far longer than that of RIA reagents.

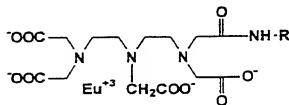
The present invention will now be described herein below with reference to an example of the assay method of CRP concentration by a dissociation-enhanced lanthanide fluorescent immunoassay (DELFI) method [Guzaeva, T. V., et al., (1993) Immunology Lett. 35, 285-290] using lanthanides, particularly Eu³⁺.

In order to label PC with Eu³⁺, the method described in the report of Saito, K., et al. (Saito, K., et al., (1998) Anal. Biochem. 258, 311-314) is used. According to this method, in order to label protein or other amino

5 following formula:

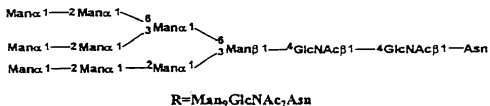


or diethylenetriamine-N¹, N², N³-pentaacetic acid (DTPA) represented by the following formula:



[in the formula, R represents amino acid or glycopeptides,
for example asparagine-bound type sugar chain as

10 represented by the following formula:



] or dianhydride thereof (DTPAda). The protein can be labeled with Eu^{3+} by binding these chelating substances by

way of the functional groups thereof to a protein and the like.

In order to label PC ($[(CH_3)_3NCH_2CH_2OPO_3H_2]^+$) with these chelating substances in accordance with the present invention, a protein having no influence on the reaction is first bound to PC to prepare a PC-protein complex. Once this is achieved, the chelating substances are bound to the protein. A protein may also be first labeled and then bound to PC. The protein is bound to PC by substituting the amino group of the protein with the PC group. Any compound which lacks an amino group can also be used provided that the compound can introduce substance chelating lanthanides.

It is required that the protein to be bound to PC is able to bind a single or multiple molecules of Eu^{3+} and must have no influence on the reaction. Any protein which satisfies these conditions can be used, and they include, but are not limited to, any available protein, for example bovine serum albumin (BSA), keyhole limpet hemocyanine (KLH), ovalbumin (OVA) and rabbit serum albumin. More preferably, BSA or KLH are used. Most preferably, BSA is used. A person having ordinary skill in the art will readily understand that substances other than proteins are also applicable for the purpose under discussion.

Using chelating substances, PC was labeled with Eu^{3+} by using DTPA in accordance with the method reported above, namely, that of Kawasaki, N., and Lee, Y. C. (1997) Anal. Biochem. 250, 260-262. PC₃₅-BSA and PC₅₅-BSA, and BSA

respectively contain 7-, 6- and 4 molecules of Eu^{3+} per one molecule of the protein. PC_{35} -BSA as used herein means a conjugate where 35 PC molecules on average are bound per one BSA molecule; PC_{98} -BSA herein means a conjugate where 58 PC molecules on average are bound per one BSA molecule. The quantity of the bound PC is almost equal to the quantity of the Eu^{3+} bound to IgG. When protein, for example anti-hCRP polyclonal rabbit IgG (molecular weight of 160 kDa) is labeled by a conventional method, 9 atoms of Eu^{3+} are bound per one molecule of the protein.

In accordance with the method of the present invention, Eu^{3+} for fluorometry is first dissociated from an initial chelating substance to form a complex, along with 1-(β -naphthyl)-3,3,3-trifluoroacetone (β -NTA). The complex emits intense fluorescence in the presence of a surfactant (the fluorescence is referred to simply as Eu^{3+} fluorescence hereinafter). The duration of the attenuation of Eu^{3+} fluorescence is longer by 10^4 -fold, as compared with polystyrene and other plastics and glass materials. Thus, the time-resolved fluorescence of Eu^{3+} in a microtiter plate can be detected with a time-resolved fluorescence microtiter plate reader (Victor, Model 1420, Wallac Co., Gaithersburg, MD) (time-resolved fluorometry). Because Eu^{3+} fluorescence can be detected following irradiation with an excitation beam for a given period of time with subsequent sufficient attenuation being conducted for the fluorescence emitted from the immobilizing phase, any adverse influence of background

fluorescence can be eliminated.

Specific conditions for detecting fluorescence (in cps) from Eu^{3+} dissociated from the chelating substance bound to the protein may be determined appropriately. As one example, Eu^{3+} fluorescence is detected by means of single photon counting, using a xenon flash lamp (1,000 Hz) for a total counting time of 1 second, with a 50- μs delay time and a 250- μs counting time per flash ($\lambda_{\text{ex}} = 340 \text{ nm}$; $\lambda_{\text{em}} = 615 \text{ nm}$), after 15-min incubation in a 200 μl /well enhancement solution [Hemmila, I., et al., (1984) Anal. Biochem. 137, 335-343] at ambient temperature. For CRP assay and PC-binding assay by the time-resolved fluorescence immunoassay (TR-FIA) method, the value of the CRP concentration in 20 mM EDTA-TBST was used as a background value.

When Eu^{3+} dissociation hardly occurs due to the intense binding of Eu^{3+} to the chelating substance, the labeled sample may be recovered from the plate, and then the sample may be burned, to modify all the organic materials therein into ashes containing only inorganic salts, to thereby enable assay of the Eu^{3+} concentration to be effected. In determining Eu^{3+} concentration by means of ash preparation, a method essentially established for determining organic phosphoric acid is used [Ames, B. N. and Dubin, D. T., (1960) J. Biol. Chem., 235, 769-758]. The ash sample is again dissolved by means of incubation in 1.5 ml of 0.1 M acetic acid at 55°C for 30 minutes. The Eu^{3+} fluorescence in an appropriate volume of the

dissolved sample (corresponding to 20 to 200 fmol Eu^{3+}) is then able to be detected by the DELFIA method. Based on the results, the Eu^{3+} concentration can be assayed accurately even after ash preparation [Saito, K., et al.,
5 (1998) Anal. Biochem. 258, 311-314].

Sandwich assay methods for determining CRP concentration in samples by using various labeling means are broadly grouped into an antibody-antibody method (A) as a sandwich assay using two species of antibodies and
10 sandwich assay methods [(B) and (C)] using PC (see Fig. 1).

The method for determining CRP concentration by the antibody-antibody method (A) comprises the steps of:

- i) immobilizing an anti-CRP antibody on a plastic
15 plate;
- ii) reacting a sample solution in which the CRP concentration is intended to be assayed, with the anti-CRP antibody; and
- iii) reacting a labeled anti-CRP antibody with the
20 sample solution to thereby determine the CRP concentration in the sample [Fig. 1 (A)].

The inventors have nevertheless found that CRP present in samples obtained from humans, particularly serum CRP can not be accurately assayed by CRP assay using
25 the antibody-antibody method (A). The reason is probably that in human serum amyloid A (hSAA) and P(hSAP) may react with anti-CRP antibodies. More specifically, hSAA and hSAP are fairly homologous to human CRP (66% and 70%,

respectively) [Agrawal, A., et al., (1994) in Acute Phase Proteins-Molecular Biology, Biochemistry and Clinical Applications-(Mackiewicz, A., et al., eds.), pp. 79-92,. CRC Press, London; Shrive, A. K., et al., (1996) Nature
5 Struc. Biol. 3, 346-354; Kilpatrick, J., and Volanakis, J. (1991) Immunol. Res. 10, 43-53]. Accordingly, hSAA and hSAP may bind to the anti-CRP antibodies to be used for the assay. When used, anti-CRP polyclonal antibodies particularly involve difficulty in the elimination of the
10 influence of the pentraxins contained in serum.

In accordance with the present invention, therefore, a sandwich assay method using only one anti-CRP antibody species and PC in place of the other antibody species is employed, so as to provide an assay method which is not
15 subject to any of the aforementioned drawbacks. The invention encompasses two types of sandwich assay methods using PC, namely antibody-PC method (B) and PC-antibody method (C). The antibody-PC method (B) comprises the steps of:

- 20 i) immobilizing an anti-CRP antibody on a plastic plate;
- ii) reacting a sample solution in which the CRP concentration is intended to be assayed, with the anti-CRP antibody; and
- 25 iii) reacting a labeled PC with the sample solution to determine the quantity of the label bound to the immobilizing phase to determine the CRP concentration in the sample [Fig. 1 (B)]. Alternatively, the PC-antibody

method (C) comprises the steps of:

i) immobilizing PC on an immobilizing phase such as a plastic plate;

- ii) reacting a sample solution in which the CRP concentration is intended to be assayed with PC; and
- iii) reacting an anti-CRP antibody with the sample solution to assay the quantity of the label bound to the immobilizing phase and thereby to determine the CRP concentration in the sample [Fig. 1 (C)].

10 In accordance with the present invention, reaction between CRP and PC is performed at least once by such a sandwich method. Because PC is not reactive with other pentraxin family members, unlike anti-CRP antibodies, the influence of undesirable reactions of PC with hSAA and

15 hSAP, which is disadvantageous for the antibody-antibody method, can be prevented.

In accordance with the present invention, any of the sandwich assay methods using PC may be satisfactorily used. So as to enhance assay sensitivity, the antibody-PC

20 method (B) is more preferably used. The PC-antibody method (C) has a drawback in that it causes a reduction in assay sensitivity, because serum lecithin blocks the PC-binding site of CRP in the presence of calcium at the reaction stage between the PC on the immobilizing phase

25 and the CRP in a serum sample. While, according to the antibody-PC method (B), the inhibition of the reaction due to serum lecithin and calcium can be prevented because PC is able to interact with CRP after washing the

immobilizing phase after the CRP in a serum sample is bound to the anti-CRP antibodies bound to the immobilizing phase. After the anti-CRP antibodies on the immobilizing phase react with a serum sample, the resulting

- 5 immobilizing phase is preferably washed with an EDTA solution. By using the antibody-PC method (B), the minimum detection limit of a sample CRP concentration can be lowered to 420 fmol. It is readily understood that PC is used after being labeled for the antibody-PC method
- 10 (B).

Effects

- The novel method for determining CRP of the present invention enables accurate assay of not only serum CRP concentration during an acute phase reaction such as in
- 15 the occurrence of infection, inflammation and tissue damage, but also serum CRP concentration within a normal range. The novel method for determining CRP of the present invention further allows detection of serum CRP within a normal range with a high degree of sensitivity in
- 20 a rapid fashion in clinical diagnosis. In addition, because the volume of a sample required for the assay is small (100 μ l per well), valuable serum samples can be saved and can thereby be subjected to the multiple assays thereby further improving accuracy.

Examples

The materials used in the following examples are as follows.

Recombinant human C-reactive protein (rhCRP)

expressed in Escherichia coli and anti-hCRP polyclonal rabbit IgG were obtained from Oriental Yeast Co., Ltd. (Japan). Phosphorylcholine (PC) chloride (calcium salt), L- α -phosphatidylcholine (L- α -lecithin derived from egg yolk), potassium hydrogen phthalate, Triton X-100 and human AB-type female sera were obtained from Sigma Chem. Co. (St. Louis, MO). Rat serum was obtained from Pel-Freeze Co. (Rogers, AR). Chelex 100 resin (sodium form) was obtained from Bio-Rad (Hercules, CA). N¹-(p-
10 isothiocyanatebenzyl)-diethylenetriamine-N¹, N², N³, N³-tetraacetic acid (DETATA-ITC) was obtained from Wallac (Gaithersburg, MD); and a 96-well microplate (cat. #21-377-204) was obtained from Fisher Scientific (Springfield, NJ). Europium nitrate, 1-(β -naphthoyl)-3,3,3-
15 trifluoroacetone, trioctylphosphine oxide (TOPO), diethylenetriamine-N¹, N², N³-pentaacetic acid (DTPA) and its dianhydride (DTPAda) were obtained from Aldrich Chem. Co. (Milwaukee, WI). Polyvinylbenzyl lactonoylamide (PVLA) was a gift from Dr. Kazukiyo Kobayashi (Nagoya
20 Univ., Japan). As PC conjugates of PC₇-, PC₃₅- and PC₅₈-BSA, and bovine serum albumin (BSA), those produced by the previously reported method [Stults, N., et al., (1987) Anal. Biochem. 161, 567-573] were used.

Example 1: Comparison of assayed CRP concentrations in sera, individually using the antibody-antibody method, the PC-antibody method and the antibody-PC method

Buffers and dissociation enhancement solution

Two types of buffers for use in CRP assay were 1)

TBS (20 mM Tris-HCl, pH 7.6 containing 0.15 M NaCl and 10 mM CaCl_2) and ii) TBST (TBS buffer containing 0.02% Tween 20).

The dissociation enhancement solution was composed of 15 μM β -NTA, 50 μM TOPO and 0.1% (w/v) Triton X-100 in 0.1 M acetic acid adjusted to pH 3.2 using potassium hydrogen phthalate [Hemmila, I., et al., (1984) Anal. Biochem. 137, 335-343].

Microtiter plate coating

10 The anti-CRP IgG antibody coated on the plate was increased in quantity, following an increase in the IgG concentration up to 5 $\mu\text{g/ml}$. Above this level, no increase in the coated quantity was observed. For the assay on microplates and the binding assay, thus, the
15 antibody of 500 ng was left to stand in wells for coating.

Because it was difficult to immobilize PC-BSA without modification of the microtiter plate, PC-BSA was immobilized on the wells of a microtiter plate by using the PVLA method [Suzuki, N., et al., (1997) Anal. Biochem.
20 247, 412-416]. PVLA (2 mg) was suspended in 1 ml of water by ultrasonication; the resulting PVLA suspension of 40 μl was added to each well, for overnight incubation at 4°C. 1 mM NaIO_4 solution was added in 50 μl -portions to each well, and the plate was incubated in darkness at ambient
25 temperature for one hour. After the plate was washed, the PC-BSA solution (100 ng protein in 40 μl of water) was added to each well, followed by mixing with 5 μl of 2% (v/v) borane-pyridine complex, for incubation at 37°C for

2 hours. The wells were blocked through incubation with 1M glycylglycine (40 μ l) at 37°C for 4 hours, and then followed by incubation with 200 μ l of TBS containing 2% (w/v) BSA at 4°C overnight. After the added solutions were removed at the individual stages of the following method, the wells were washed six times with 200 μ l of TBS.

To immobilize PC-BSA using the PVLA method, the level of coating was increased to 100 ng of the PC-BSA used per well, depending on the quantity of PC-BSA used. Thus, 100 ng of PC-BSA was routinely used for the PVLA method.

After preliminary tests were carried out, the level of Eu^{3+} -labeled anti-CRP IgG (20 ng IgG, 1 pmol Eu^{3+} /well) or PC₃₅-BSA (20 ng BSA, 1.8 pmol Eu^{3+} /well) was selected, so that the background count could be minimized.

CRP assay by DELFIA method

The principles of the three assay methods are shown in Fig. 1. Unless otherwise stated, the reagent solutions were discarded at the individual stages described, followed by washing of the wells six times with 200 μ l TBST. Eu^{3+} fluorescence was finally detected by the DELFIA method previously reported [Guzaeva, T. V., et al., (1993) Immunol. Lett. 35, 285-290].

25 (A) Antibody-antibody method

100 μ l of TBS containing the anti-CRP antibody (500 ng) was added to each well, for overnight incubation at 4°C. Each well was blocked through incubation in 200 μ l

of 2% (w/v) BSA (in TBS) at ambient temperature for 2 hours; after discarding the BSA solution, the well was washed six times with 200 μ l of TBS. 100 μ l each of TBST samples containing variable quantities of CRP was applied to the wells coated with the antibody, followed by incubation at ambient temperature for 2 hours. After discarding the CRP solution, 100 μ l of a TBST buffer solution containing 20 ng of Eu^{3+} -labeled anti-CRP antibody (9 Eu^{3+} per IgG) was added to the wells. Subsequently, the plate was incubated at ambient temperature for 2 hours, to detect Eu^{3+} fluorescence.

(B) Antibody-PC method

A microtiter plate coated with the anti-CRP antibody and blocked with BSA was prepared as described above. 100 μ l each of TBST containing CRP at variable quantities were left to stand in the wells, which were incubated at ambient temperature for 2 hours. After the CRP solution was discarded from the wells, 100 μ l of TBST containing Eu^{3+} -labeled PC-BSA was added to the wells, for incubation at ambient temperature for 2 hours. CRP treated with TBST containing 20 mM EDTA, and BSA labeled with Eu^{3+} were added to the coated wells, which were used as a background and as a standard.

(C) PC-antibody method

So as to immobilize PC_{58} - or PC_7 -BSA solution (40 μ l containing 100 μ g protein and PC of about 120 pmol or 15 pmol) in water, the solution was left to stand in the PVLA-coated well. After the wells were coated with PC-BSA

and blocked with BSA, they were then washed six times with TBS (200 µl). 100 µl each of TBST containing various concentrations of CRP were introduced in the wells, for incubation at ambient temperature for 2 hours. After discarding the CRP solutions, the Eu³⁺-labeled anti-CRP antibody (20 ng in 100 µl of TBST) was added, and the plate was then incubated at ambient temperature for 2 hours.

Comparison of values of CRP concentration assayed

10 CRP assays by the antibody-antibody method (A) and the PC-antibody method (C) individually exerted two-phase standard curves (10 to 60 ng and 100 to 200 ng, respectively) (Fig. 2b). Alternatively, the results of the CRP assay by the antibody-PC method (B) were on a line 15 within a range of 5 to 500 ng CRP/ml (Fig. 2a). The range was far wider than the ranges shown in Fig. 2b.

The antibody-antibody method (A) and the antibody-PC method (B) were not influenced by the presence of serum. rhCRP added to sera was not accurately determined by the 20 PC-antibody method (C) (Fig. 3). Despite the presence or absence of BSA or PdC, rhCRP at known quantities in 100-fold diluted sera was effective in bringing about results expected from the antibody-antibody method (A) and the antibody-PC method (B). However, for the antibody-antibody 25 method (A), the acceptable results with negligible interference were obtained only within a limited range (Fig. 4).

The DELFIA technique has enabled the development of

a highly sensitive CRP assay method, as regards CRP assay. The degree of sensitivity and accuracy of the antibody-PC method (B) using a plate coated with the anti-CRP antibody and PC-BSA labeled with Eu^{3+} are so high that 400 fmol Eu^{3+} (namely, 420 fmol CRP) can be detected. The specific binding can be recovered by removing a non-specific binding count from the total binding count (Fig. 8). The sensitivity corresponds to the range of 5 to 500 ng CRP/ml (Fig. 2) and is comparative to that of radioimmunoassay (8 to 800 ng CRP/ml) [Claus, D. R., et al., (1976) J. Lab. Clin. Med. 87, 120-128]. The standard deviation from triplet assays was less than 3%. Further, the linear range detectable by the invented method was far wider as compared with general ELISA methods [by ELISA methods, the range was 10 to 100 ng CRP/ml (Unten, S. K., and Hokama, Y. (1987) J. Clin. Lab. Anal. 1, 136-139)]. The antibody-PC method (B) directly uses PC-BSA labeled with Eu^{3+} in place of a secondary antibody, and is thus simpler. Owing to the assay sensitivity, CRP at a normal levels in serum can also be assayed even by using a serum sample in 10- to 100-fold dilution.

Example 2: PC-binding assays, individually using the antibody-antibody method, the PC-antibody method and the antibody-PC method.

25 Buffers and dissociation enhancement solution

The two types of buffers and the dissociation enhancement solution used for PC-binding assay were prepared in the same manner as those used for CRP assay.

Microtiter plate coating

The microtiter plate used for PC-binding assay was prepared by using anti-CRP IgG antibody or PC-BSA in the same manner as the plate used for CRP assay.

5 Quantitative precipitation of CRP

Quantitative precipitation reaction was carried out as follows: variable quantities of PC₅₉-BSA in TBS was added to CRP (50 µg) in TBS to a final volume of 50 µl. After incubation at 37°C for one hour, the resulting
10 mixture was left to stand at 4°C for 48 hours and then centrifuged (10,000 g x 5 minutes). The precipitate was washed three times with cold TBS and was then dissolved in 0.5 M NaOH (50 µl).

Protein concentration in the precipitate was
15 determined on the basis of bicinchoninic acid (BCA) assay with a Pierce kit (Rockford, IL) by using rhCRP as a CRP standard or by using BSA as a PC-BSA standard [Smith, P. K., et al., (1985) Anal. Biochem. 193, 76-85]. When BSA is used as a standard for CRP assay, a coefficient of 0.84
20 should be used so as to assay accurately CRP concentration by the BCA method. Phosphoric acid was measured by using phosphorylcholine as a standard by the organic phosphoric acid analysis method of Ames, et al. [Ames, B. N., and Dubin, D. T. (1960) J. Biol. Chem. 235, 765-775].

25 Interference by BSA, lecithin (PdC) and human sera

By using the aforementioned three methods, namely the antibody-antibody method (A), antibody-PC method (B) and PC-antibody method (C), CRP was assayed in a serum in

100-fold dilution with TBST. The rhCRP (10 to 40 ng/well) was added to the diluted serum, and the resulting mixture was left to stand in wells for assay. The influence of the presence of serum on these methods was compared using
5 rhCRP (10 ng) as a standard. Similarly, TBST containing BSA (10 mg/ml) or PdC (1 mg/ml) was added to a solution containing variable quantities of rhCRP in order to examine the interference of said TBS containing TBST and PdC. As a blank test, CRP was diluted with TBST
10 containing 20 mM EDTA.

PC-binding assay by DELFIA method

The three assay methods used for PC-binding assay were identical to the three methods used for CRP assay. And as with CRP assay, Eu^{3+} fluorescence was finally
15 detected according to the DELFIA method.

PC-binding assay

CRP concentration in mol is expressed as the pentamer (molecular weight of 118,000).

(i) Precipitation method

20 The CRP activity to bind PC was assayed through quantitative precipitation by using 10 to 80 μg PC_{50} -BSA and 50 μg CRP to determine the protein and phosphoric acid in the precipitate and assay the binding activity of CRP (Fig. 5). The precipitate reached a plateau at 20 μg of
25 the PC-BSA level, while 10 nmol phosphoric acid was observed in 70 μg of the precipitate (as BSA).

(ii) Microtiter plate method

Using the antibody-PC method (B) and the PC-antibody

method (C), the PC-binding activity was assayed, using 20 ng of a Eu^{3+} -labeled probe, within a CRP range of 5 to 100 ng per well. In the case that PC-BSA or PdC was directly coated on plastic wells, no material coated (with PC-BSA or PdC) at a sufficiently high level for the assay of the binding activity of CRP was recovered, even when a Eu^{3+} -labeled probe was used (the data not shown). Thus, a PC-BSA immobilized plate was prepared by using the PVLA method.

10 When BSA (10 mg/ml) was present, the antibody-PC method (B) or the PC-antibody method (C) was never influenced; when 1 mM PdC (PdC concentration in serum) or diluted serum was present, the PC-antibody method (C) was influenced but the antibody-PC method (B) was not
15 influenced (Fig. 3).

 It is surmised that the interference of the serum components in the PC-antibody method (B) may be due to the reaction of PdC mainly with the PC-binding site of CRP (Fig. 3). In fact, it is known that CRP precipitates together with lecithin in the presence of calcium [Hokama, Y., et al., (1974) Clin. Chem. Acta 50, 53-62]. So as to prevent such PdC interference, therefore, it was indicated that the antibody-PC method (B) which enables anti-CRP antibodies with no relation to the PC-binding activity to
25 capture CRP from serum samples, was preferably used. Further, after a sample containing CRP was applied to the plate, additionally, the plate was preferably washed with an EDTA solution. This is intended to remove inhibitory

substances in serum from the PC-binding site (data not shown).

Example 3: CRP activity to bind to PC

Preparation of Ca-free PC

5 Phosphorylcholine chloride (calcium salt) was
suspended in water, together with 100 ml of Chelex 100
(sodium type); and the resulting suspension was agitated
overnight at ambient temperature. The resin was filtered
under aspiration. On the filter, the resin was then
10 washed in water. Organic phosphoric acid in the resulting
aqueous PC solution (sodium salt) was analyzed and freeze-
dried into powder.

Inhibition of PC-binding activity by PC

 Various amounts of PC were incubated in a total 100
15 μ l volume of each of TBST solutions containing 2, 5, 10,
50 and 100 ng CRP, at 37°C for 2 hours. The resulting
mixtures were left to stand in wells immobilized with PC₅₈-
BSA, for incubation at ambient temperature for 2 hours.
The CRP activity to bind to PC was assayed by using anti-
20 CRP, as described above.

CRP activity to bind to immobilized PC-BSA

 The anti-CRP antibody (IgG) was bound at 5.6 f mol
and 2.2 fmol per well to PC₅₈-BSA-coated well and PC₅₈-BSA-
coated well, respectively (Fig. 6). The inhibition
25 potency of PC against the CRP activity to bind to the PC-
BSA was assayed at various amounts of CRP. When CRP was
at 2, 5, 10, 20, 50 and 100 ng, the PC concentration at
50% inhibition (IC₅₀) was 5, 11, 16, 17, 27 and 37 μ M

(Fig. 7) respectively, when the PC-antibody method (C) was used.

The antibody-PC method (B) has additional advantages. Most of current CRP assay methods singly
5 depend on anti-hCRP antibody. Because human serum amyloid A (hSAA) and P(hSAP) are of the pentraxin family and with considerable homology to hCRP (66% and 70%, respectively) [Agrawal, A., et al., (1994) in Acute Phase Proteins-
Molecular Biology, Biochemistry and Clinical Applications-
10 (Machewicz, A., et al., eds.), pp. 79-92, CRC Press, London; Shrive, A. K., et al., (1996) Nature Struc. Biol. 3, 346-354; Kilpatrick, J., and Volanakis, J. (1991) Immunol. Res. 10, 43-53], anti-hCRP antibodies possibly react with these pentraxins when the antibodies are
15 polyclonal antibodies and used as primary antibodies for the assay. Meanwhile, only CRP is reactive with PC-BSA [Eriksen, N., et al., (1994) in Acute Phase Proteins-
Molecular Biology, Biochemistry and Clinical Applications-
(Machewicz, A., et al., eds.), pp. 93-106, CRC Press,
20 London]. According to the antibody-PC method (B), therefore, only CRP is assayed through specific reaction with PC-BSA labeled with Eu^{3+} during the final stage of the method.

At saturation precipitation when 20 μg PC_{58} -BSA was
25 added, the precipitate contained 0.17 nmol PC_{58} -BSA (10 nmol PC in total) and 0.37 nmol CRP, and the chemical amount of PC_{58} -BSA bound to CRP was 1:2.2 (as the ratio of PC_{58} -BSA : CRP; Fig. 5). The PC-BSA immobilized by the

PVLA method was approximately calculated to be 5 to 6 fmol
(2.4×10^5 to 2.6×10^5 cps) per well based on the Eu^{3+}
count according to the PC-antibody method (C), provided
that the ratio of PC-BSA : CRP: anti-CRP IgG in the
5 complex was 1 : 1: 1 (Fig. 6).

The PC-binding affinity of CRP was assayed by
equilibrium dialysis at 15°C using radioactively labeled PC
and capillary electrophoresis at 21°C. The results were K_d
= 6.3 μM [Anderson, J. K., et al., (1978) Fed. Proc. 37,
10 1495] and K_d = 17.7 μM [Heegaard, N. H. H., and Robey, F.
A. (1993) J. Immunol. Methods 166, 103-110]. Since PC
inhibited CRP at 0.2, 0.4, 0.9, 1.7, 4.2 and 8.5 pM at 37°C
by using the PC-antibody method (C), the IC_{50} of PC was 5,
11, 16, 17, 27 and 37 μM in the order, consequently (Fig.
15 7). The ligand concentration at 50% inhibition is
frequently used to approximately express a dissociation
constant when both the receptor- and ligand concentrations
are below 1% of the actual K_d [Chang, K.-J., et al., (1975)
Biochem. Biophys. Acta 406, 294-303]. Although the amount
20 of PC-BSA immobilized on the well is not known, the amount
occupies only 0.2% of the reported K_d value, even if the
total amount of PC-BSA is immobilized. Additionally, the
CRP concentration is below 0.01% of the K_d value. Thus,
the IC_{50} value can approximately express binding constant
25 (K_d). In such a case, the K_d value (5 to 37 μM)
approximately calculated in such a manner is consistent
with the results of the equilibrium dialysis and the
capillary electrophoresis (6.3 to 17.7 μM).

CLAIMS

1. An in vitro method for determining a concentration of C-reactive protein (CRP) in a sample, using labeled phosphorylcholine (PC).
2. The method for determining a concentration of CRP according to Claim 1, wherein the sample containing CRP is a liquid derived from a human being.
3. An in vitro method for determining a concentration of CRP in a sample comprising the step of:
 - (i) binding an anti-CRP antibody to an immobilizing phase;
 - (ii) reacting a sample solution with the antibody bound to the immobilizing phase to bind the CRP in the sample to the antibody on the immobilizing phase;
 - (iii) reacting a labeled PC with the CRP bound to the antibody on the immobilizing phase; and
 - (iv) detecting the signal from the labeled PC bound on the immobilizing phase; and
 - (v) determining the concentration of CRP in the sample on the basis of the intensity of the signal.
4. The method according to any one of Claims 1 to 3, wherein PC is labeled with a radioactive labeling means or a non-radioactive labeling means.
5. The method according to Claim 4, wherein the non-radioactive labeling means is a lanthanide.
6. The method for determining a concentration of CRP according to Claim 5, wherein the labeling of PC with a lanthanide is carried out by labeling another substance

bound to PC.

7. The method according to Claim 5 or 6, wherein the lanthanide is Eu^{3+} .

8. A kit for determining a concentration of CRP in a sample, comprising

(i) an immobilizing phase where an anti-CRP antibody is immobilized; and

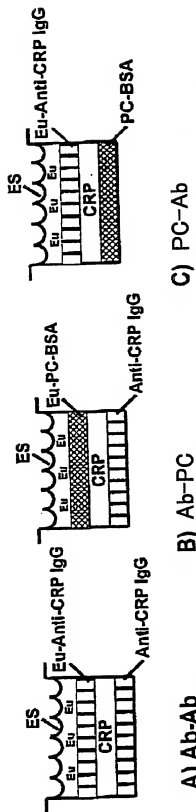
(ii) PC labeled with a lanthanide.

9. The kit according to Claim 8, wherein the lanthanide is Eu^{3+} .

ABSTRACT

C-reactive protein is an acute phase protein reactive to extraneous stimuli with a high degree of sensitivity. By comparing the level of acute phase
5 protein during bacterial infection, inflammation and post-surgery prognosis and an accurately assayed CRP level under normal conditions, symptoms can be diagnosed with more accuracy. However, serum CRP concentration at a normal level can not be accurately assayed by conventional
10 methods due to the low sensitivity. In conventional methods, CRP levels are simply expressed as "negative", "positive" or "elevated". The subject invention relates to a novel method for assaying C-reactive protein (CRP) in a sample. More specifically, the invention relates to a
15 novel method for determining a CRP concentration in a sample, using the specific binding between phosphorylcholine (PC) and CRP. Still more specifically, the invention relates to a novel method for determining a CRP concentration in a sample, comprising a sandwich assay
20 process of assaying CRP in a sample as immobilized on an immobilizing phase by using labeled PC. In accordance with the subject invention, radioisotopes and fluorescent dyes (fluorescein, lanthanide and the like) may satisfactorily be used to label PC; more preferably
25 fluorescent dyes, and most preferably lanthanide may be used as such fluorescent substances.

Fig. 1



Steps	Procedures		
	(A) Ab-Ab	(B) Ab-PC	(C) PC-Ab
Coating	Anti-CRP antibody	Anti-CRP antibody	PC-BSA
Analyte-binding	CRP	CRP	CRP
Probe-binding	Anti-CRP antibody	PC-BSA	Anti-CRP antibody

* ES: enhancement solution.

Fig. 2

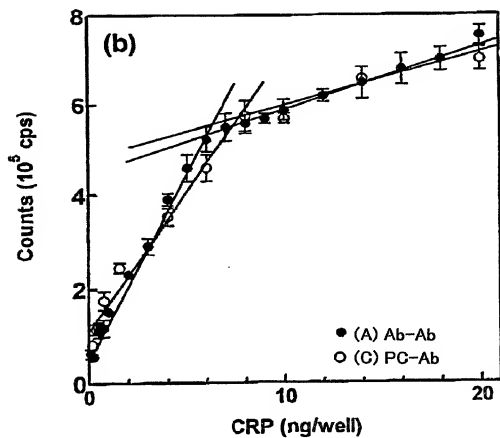
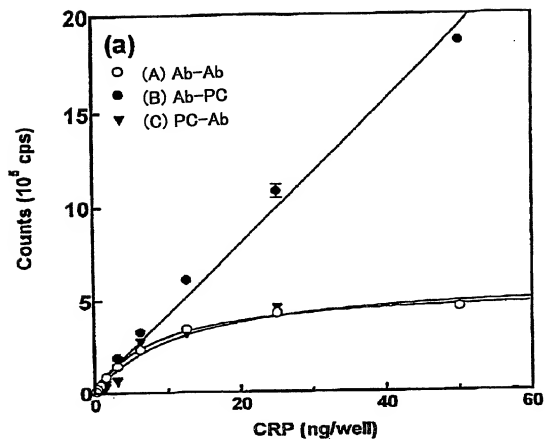


Fig. 3

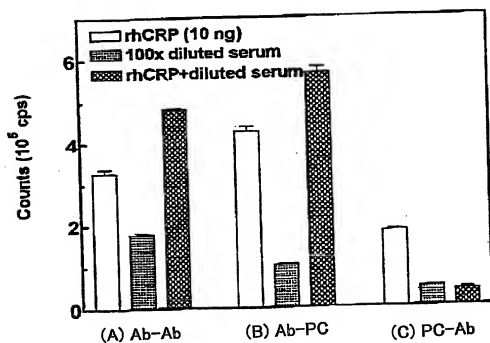


Fig. 4

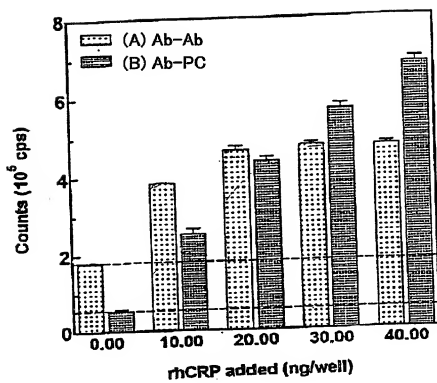


Fig. 5

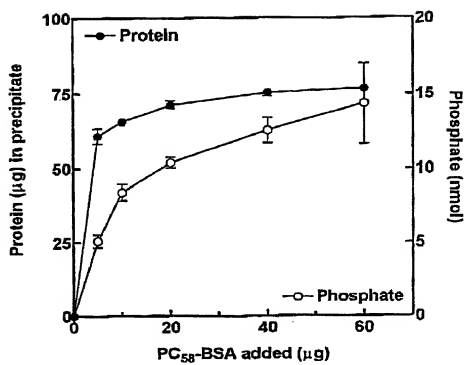


Fig. 6

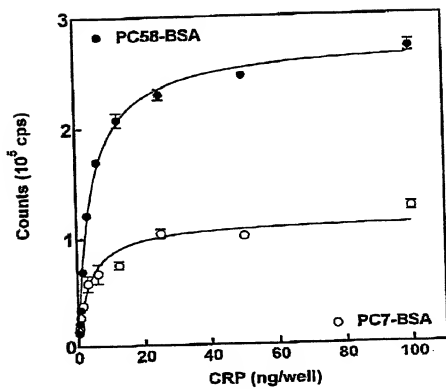


Fig. 7

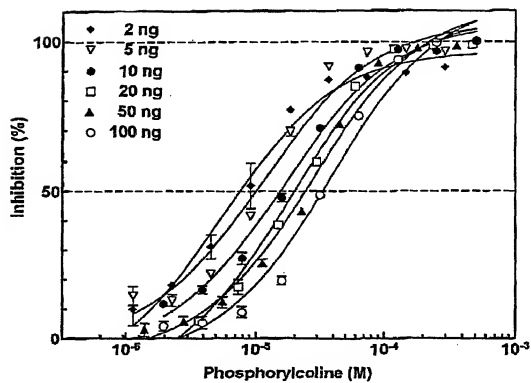
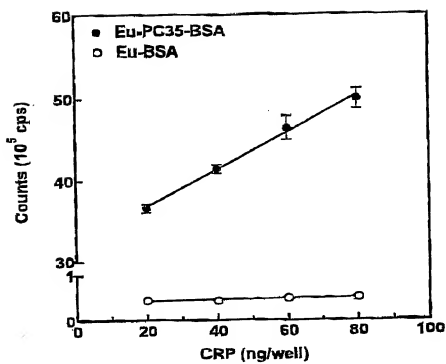


Fig. 8



RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHOD FOR DETERMINING CRP USING PHOSPHORYLCHOLINE

the specification of which (check applicable box(es)):

☒ is attached hereto

☐ was filed on _____

☐ was filed as PCT International application No. _____ on _____

and (if applicable to U.S. or PCT application) was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed, if no priority is claimed, before the filing date of this application:

Prior Foreign Application(s):

Application Number
55352/1999

Country
Japan

Day/Month/Year Filed
3/3/1999

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

Application Number

Date/Month/Year Filed

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

Prior U.S./PCT Application(s):

Application Serial No.
PCT/JP00/01268

Day/Month/Year Filed
3/3/2000

Status: patented
pending, abandoned
pending

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And I hereby appoint **NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed)**, and the following attorneys thereof (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon, 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Jeffrey H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr. 29366; Thomas E. Byrne, 32205; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Alan M. Kagen, 36178; William J. Griffin, 31260; Robert A. Molan, 29834; B. J. Sadoff, 38663; James D. Berquist, 34776; Updeep S. Gill, 37334.

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RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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(Zip Code)
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Residence: (city) _____ (state/country) _____
Post Office Address: _____
(Zip Code)
8. Inventor's Signature: _____ Date: _____
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(Zip Code)
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(Zip Code)
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Post Office Address: _____
(Zip Code)
11. Inventor's Signature: _____ Date: _____
Inventor: _____
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Residence: (city) _____ (state/country) _____
Post Office Address: _____
(Zip Code)
12. Inventor's Signature: _____ Date: _____
Inventor: _____
(first) MI (last) (citizenship)
Residence: (city) _____ (state/country) _____
Post Office Address: _____
(Zip Code)

DECLARATION OF TRANSLATION

I, Kazuyo IIZUKA, a member of the staff of the YUASA AND HARA, (Section 206, New Ohtemachi Bldg.,) 2-1, Ohtemachi 2-chome, Chiyoda-ku, Tokyo, Japan, do hereby solemnly and sincerely declare as follows:

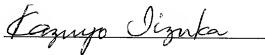
1. That I am acquainted with the Japanese and English languages,
2. That I am the translator of the accompanying certified official transcript from the Family Register.
3. That the attached document in English is true and correct translation to the best of my knowledge and belief.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated this 30th day of October, 2000

Full name of the translator: Kazuyo IIZUKA

Signature of the translator:



Post Office Address: c/o YUASA AND HARA, Section 206,
New Ohtemachi Bldg., 2-1,
Ohtemachi 2-chome, Chiyoda-ku,
Tokyo, JAPAN

FAMILY REGISTER

Permanent Address: 37, Nigishimasato-cho, Kumano-shi,
Mie-ken

Name : KAWAGUCHI, Kichitaro

This family register was compiled on September 30, 1972
due to the notification of marriage.

Father: KAWAGUCHI, Keiichi
Eldest son
Mother: Yoshiko
HUSBAND: ~~Kichitaro~~
Date of Birth: October 17, 1941

Born on October 17, 1941 in Tennouji-ku, Osaka-shi, reported by father on the 29th of the same month. The notification was received by the village headman of Kasuga-mura, Mishima-gun, Osaka on the 31st day of the same month and entered in the Family Register.

Married to Eiko OHASHI on September 25, 1972, and a notification of the marriage was given by the mayor of Nara-shi on 30th day of the same month, and entered into the Family Register transferring from the Family Register of Keichi KAWAGUCHI at 37, Nigishimasato-cho, Kumano-shi, Mie-ken.

Died on October 12, 1999, 1:00 p.m. in Fujishiro-machi, Kitasouma-gun, Ibaraki-ken and the notification was given on the 13th day of the same month by a relative, Eiko KAWAGUCHI, forwarded by the mayor of the same town on the 15th day of the same month and deleted from the Family Register.

Father: OHASHI, Harukichi
Eldest daughter
Mother: Yoko
WIFW : Eiko
Date of Birth: September 7, 1946

Born on September 7, 1946 in Kamakura-shi, Kanagawa-ken, reported by father on the 12th of the same month. The notification was given by the mayor of the

same city on the 1st day of October and entered in the Family Register.

Married to Kichitaro KAWAGUCHI on the 25th day of September 1972, and a notification of the marriage was given by the mayor of Nara-shi on the 30th day of the same month, and entered into the Family Register by transferring from the Family Register of Harukichi OHASHI at 159, Numabukuro 1-chome, Nakano-ku, Tokyo.

Her husband died on the 12th day of October 1999.

Father: KAWAGUCHI, Kichitaro
Eldest son
Mother: Eiko
Takehiko
Date of Birth: October 17, 1973

Born on October 17, 1973 in Sacramento, California, U. S. A., reported by father on the 22nd day of the same month without identifying nationality. The notification was forwarded by the Japanese General Consul at San Francisco on the 25th day of November 1973 and entered in the Family Register.

Father: KAWAGUCHI, Kichitaro
Eldest daughter
Mother: Eiko
Yayoi
Date of Birth: March 2, 1975

Born on March 2, 1975 in Nara-shi reported by father on the 11th day of the same month. The notification was forwarded by the mayor of the same city on the 17th day of the same month and entered in the Family Register.

This is to exemplify that this is a true and correct copy of the original Family Register.

July 17, 2000

KAWAKAMI, Kanji (seal)
Mayer of Kumano-shi, Mie-ken